

## Isolation of a primate embryonic stem cell line

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**ABSTRACT** Embryonic stem cells have the ability to remain undifferentiated and proliferate indefinitely *in vitro* while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. Here we report the derivation of a cloned cell line (R278.5) from a rhesus monkey blastocyst that remains undifferentiated in continuous passage for >1 year, maintains a normal XY karyotype, and expresses the cell surface markers (alkaline phosphatase, stage-specific embryonic antigen 3, stage-specific embryonic antigen 4, TRA-1-60, and TRA-1-81) that are characteristic of human embryonic carcinoma cells. R278.5 cells remain undifferentiated when grown on mouse embryonic fibroblast feeder layers but differentiate or die in the absence of fibroblasts, despite the presence of recombinant human leukemia inhibitory factor. R278.5 cells allowed to differentiate *in vitro* secrete bioactive chorionic gonadotropin into the medium, express chorionic gonadotropin  $\alpha$ - and  $\beta$ -subunit mRNAs, and express  $\alpha$ -fetoprotein mRNA, indicating trophoblast and endoderm differentiation. When injected into severe combined immunodeficient mice, R278.5 cells consistently differentiate into derivatives of all three embryonic germ layers. These results define R278.5 cells as an embryonic stem cell line, to our knowledge, the first to be derived from any primate species.

Embryonic stem (ES) cells, derived from preimplantation embryos (1, 2), and embryonic germ (EG) cells, derived from fetal germ cells (3, 4), are undifferentiated, immortal cells capable of differentiating into derivatives of all three embryonic germ layers. Well-characterized ES and EG cells have been derived only from rodents (1, 2, 5, 6). Pluripotent cell lines have been derived from preimplantation embryos of several non-rodent species (7–10), but the developmental potentials of these cell lines remain poorly characterized. Mouse ES cells remain undifferentiated through serial passages when cultured in the presence of leukemia inhibitory factor (LIF) and differentiate in the absence of LIF (11). Mouse ES cells injected into syngeneic mice form teratocarcinomas that exhibit disorganized differentiation, with representatives of all three embryonic germ layers. Mouse ES cells combined with normal preimplantation embryos as chimeras and returned to the uterus participate in normal development (12). Because mouse ES cells can contribute to functional germ cells in chimeras, specific genetic changes can be introduced into the mouse germ line through the use of ES cell chimeras (13).

The mechanisms controlling differentiation of specific lineages can be studied with mouse ES cells grown *in vitro*; however, significant differences between early human and mouse development suggest that human development will be more accurately represented by primate ES cells. For example, human and mouse embryos differ in the timing of embryonic genome expression (14), in the structure and function of the

fetal membranes and placenta (15), and in formation of an embryonic disc instead of an egg cylinder. Human embryonic carcinoma (EC) cells, which are pluripotent, immortal stem cells from teratocarcinomas, provide an important *in vitro* model for understanding human differentiation (16). Some EC cell lines can be induced to differentiate in culture (17), which results in the loss of specific cell surface markers [stage-specific embryonic antigen 3 (SSEA-3), SSEA-4, TRA-1-60, and TRA-1-81] and the appearance of new markers (16). When pluripotent human EC cells are injected into immunocompromised mice, they form teratocarcinomas, some with derivatives of all three embryonic germ layers. However, there are limitations to the use of human EC cells in the study of development. (i) The range of differentiation obtained from human EC cell lines is more limited than that obtained from mouse ES cells and varies widely between cell lines (18). (ii) All pluripotent human EC cell lines derived to date are aneuploid (19), suggesting EC cells may not provide a completely accurate representation of normal differentiation. (iii) Ethical considerations severely restrict the study of human embryos, often making it impossible to verify that *in vitro* results have significance in the intact embryo. None of these limitations would be present with nonhuman primate ES cell lines.

Here we report the isolation of an ES cell line (R278.5) from a rhesus monkey blastocyst. This cloned cell line remains undifferentiated and continues to proliferate for >1 year in culture, maintains a normal XY karyotype, and maintains the potential to differentiate into trophoblast and to derivatives of embryonic endoderm, mesoderm, and ectoderm. The morphology, cell surface markers, and growth factor requirements of these cells differ significantly from mouse ES cells but closely resemble human EC cells.

## MATERIALS AND METHODS

**Cell Line Isolation.** Six days after ovulation, an azonal blastocyst was recovered by a nonsurgical uterine flush technique from a 15-year-old rhesus monkey (20). The trophectoderm was removed by immunosurgery (21) using a rabbit anti-rhesus spleen cell antiserum followed by exposure to guinea pig complement. The intact inner cell mass (ICM) was separated from lysed trophectoderm cells and plated on mouse embryonic fibroblasts [previously exposed to 3000 rads (1 rad = 0.01 Gy)  $\gamma$ -radiation] in medium consisting of 80% Dulbecco's modified Eagle medium (4500 mg of glucose per liter, with L-glutamine, without sodium pyruvate; GIBCO) with 20% fetal bovine serum (HyClone), 0.1 mM 2-mercaptoethanol (Sigma), 1% nonessential amino acid stock (GIBCO) (22), and 1000 units of cloned human LIF per ml (GIBCO). After 16 days of culture, a central mass of cells was removed from

Abbreviations: CG, chorionic gonadotropin; ES, embryonic stem; EC, embryonic carcinoma; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ICM, inner cell mass; LIF, leukemia inhibitory factor; RT-PCR, reverse transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SSEA, stage-specific embryonic antigen.

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epithelial outgrowths, exposed for 3 min to 0.05% trypsin-EDTA (GIBCO), gently dissociated by pipetting through a micropipette, and replated on mouse embryonic fibroblasts. After 3 weeks of growth, colonies with a morphology resembling human EC cells were selected and expanded. At five passages, individual cells were selected by micropipette and plated in individual wells of a 96-well plate (Falcon) with mouse embryonic fibroblast feeder layers. One clone with a normal karyotype (R278.5) was expanded for further analysis.

**Cell Surface Markers.** R278.5 cells grown on a layer of mouse embryonic fibroblasts were used to examine the expression of cell surface markers. Alkaline phosphatase was detected histochemically following fixation of cells with 100% ethanol using "Vector red" (Vector Laboratories) as a substrate, as described by the manufacturer. The SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 antigens were detected by immunocytochemistry with specific primary monoclonal antibodies (gifts of Peter Andrews, University of Sheffield, U.K.) (16, 23–25) and localized with a biotinylated secondary antibody and then an avidin/biotinylated horseradish peroxidase complex (Vectastain ABC system, Vector Laboratories).

**In Vitro Differentiation.** R278.5 cells were plated at low density (~5000 cells/cm<sup>2</sup> of surface area) in the absence of fibroblasts on gelatin-treated four-well tissue culture plates (Nunc) in the same medium as that used for initial cell line isolation, but with 0–10<sup>4</sup> units of added human LIF per ml (GIBCO). The resulting differentiated cells were photographed 8 days after plating.

A mouse Leydig cell bioassay (26) was used to measure luteinizing hormone/chorionic gonadotropin (CG) activity in medium conditioned for 2 days either by undifferentiated R278.5 cells (at 80% confluence on fibroblast feeder layers) or by spontaneously differentiated R278.5 cells (cultured for 2 weeks after achieving confluence on fibroblast feeders). The relative levels of the mRNAs for  $\alpha$ -fetoprotein and the  $\alpha$ - and  $\beta$ -subunits of CG relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (27) using RNA from the same undifferentiated and differentiated cells. The PCR primers for human G3PDH (Clontech) do not amplify mouse G3PDH mRNA. Primers for human  $\alpha$ -fetoprotein mRNA flank the seventh intron (5' primer, 5'-GCTGGATTGTCTGCAGGATGGGGAA; 3' primer, 5'-TCCCCTGAAGAAAATTGGTTAAAAAT) and amplify a cDNA of 216 bp. Primers for the  $\beta$ -subunit of human CG flank the second intron (5' primer, 5'-ggatcCACCGTCAACACCACCATCTGTGC; 3' primer, 5'-ggatcCACAGGTCAAAGGGTGGTCCTTGGG) (nucleotides added to the CG $\beta$  sequence to facilitate subcloning are shown in italics) and amplify a cDNA of 262 bp. The primers for the CG $\alpha$  subunit were based on sequences of the first and fourth exon of the rhesus gene (28) (5' primer, 5'-gggaattcGCAGTTACTGAGAACTCACAAG; 3' primer, 5'-gggaattcGAAGCATGTCAAAGTGGTATGG) and amplify a cDNA of 556 bp. The identity of all cDNAs was verified by sequencing (not shown).

For RT-PCR, 1–5  $\mu$ l of total R278.5 RNA was reverse transcribed, and 1–20  $\mu$ l of reverse transcription reaction was subjected to the PCR in the presence of 2.5  $\mu$ Ci of deoxycytidine 5'-[ $\alpha$ -<sup>32</sup>P]triphosphate (1 Ci = 37 GBq; DuPont). The number of amplification rounds that produced linear increases in target cDNAs and the relation between input RNA and amount of PCR product were empirically determined. Following agarose gel electrophoresis, DNA bands of interest were cut out and radioactivity was determined by liquid scintillation spectroscopy. The ratio of cpm in a specific PCR product relative to cpm of G3PDH PCR product was used to estimate the relative levels of mRNAs among differentiated and undifferentiated cells.

**Tumor Formation in Severe Combined Immunodeficient (SCID) Mice.** In the passage immediately prior to SCID mouse injection (7 months after initial derivation of R278), karyotypes of R278.5 were confirmed as euploid. Approximately 5  $\times$  10<sup>5</sup> R278.5 cells were injected either into the rear leg muscles (seven mice) or into the testis (two mice) of 8- to 12-week-old male SCID mice. The resulting tumors were fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8–15 weeks of development.

## RESULTS

The morphology and cell surface markers of R278.5 cells (Fig. 1A) more closely resembled human EC cells than mouse ES cells. R278.5 cells had a high nucleus/cytoplasm ratio and prominent nucleoli, but rather than forming compact, piled-up colonies with indistinct cell borders similar to mouse ES cells, R278.5 cells formed flatter colonies with individual, distinct cells. R278.5 cells expressed alkaline phosphatase activity and the cell surface antigens SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 2), cell surface markers characteristic of human EC cell lines (16). Although cloned human LIF was present in the medium at cell line derivation and for initial passages, R278.5 cells grown on mouse embryonic fibroblasts without exogenous LIF remained undifferentiated and continued to proliferate. R278.5 cells plated on gelatin-treated tissue culture plates without fibroblasts differentiated to multiple cell types or failed to attach and died, regardless of the presence or absence of exogenously added human LIF (Fig. 1B).

The mRNA for  $\alpha$ -fetoprotein, a marker for endoderm, increased substantially with *in vitro* differentiation (Fig. 3).  $\alpha$ -Fetoprotein is expressed by extra-embryonic (yolk sac) and embryonic (fetal liver and intestines) endoderm. Epithelial cells resembling extraembryonic endoderm were present in cells differentiated *in vitro* from R278.5 cells (Fig. 1B).

Luteinizing hormone activity, an indication of CG secretion and trophoblast differentiation, was present in culture medium collected from differentiated cells [3.89 milli-international units (mIU)/ml] but not in medium collected from undiffer-

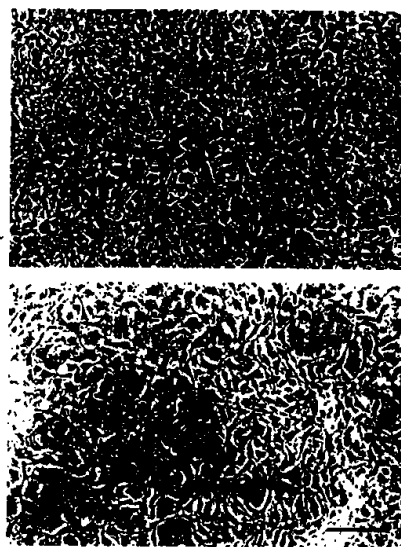


Fig. 1. Colony morphology and *in vitro* differentiation of cell line R278.5. (A) Undifferentiated R278.5 cells. Note the distinct cell borders, high nucleus to cytoplasm ratio, and prominent nucleoli. (Bar = 100  $\mu$ m.) (B) Differentiated cells 8 days after plating R278.5 cells on gelatin-treated tissue culture plastic, with 10<sup>3</sup> units of added human LIF per ml. (Bar = 100  $\mu$ m.)

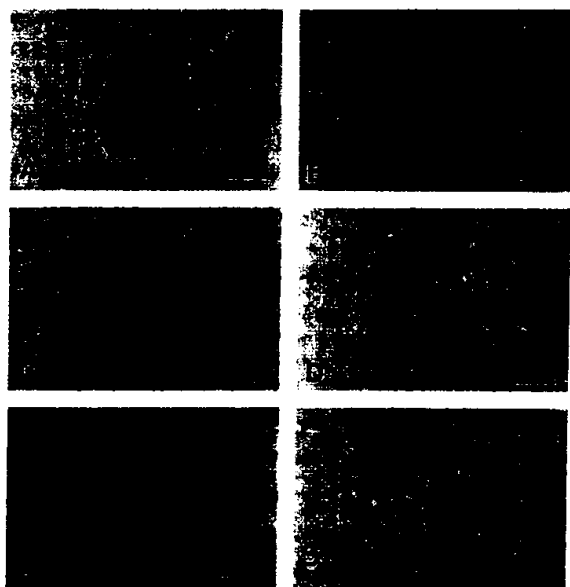


FIG. 2. Expression of cell surface markers by undifferentiated R278.5 cells. (A) Alkaline phosphatase. (B) SSEA-1. (C) SSEA-3. (D) SSEA-4. (E) TRA-1-60. (F) TRA-1-81. (Bars = 100  $\mu$ m.) SSEA-3 staining of R278.5 cells was consistently weaker than the other positive antigens, and cell staining intensity varied within and between colonies.

entiated cells (<0.03 mIU/ml). The mRNAs for the CG subunits were readily detectable in the differentiated cells, although the relative level of the CG $\beta$  subunit mRNA was considerably lower than that for the CG $\alpha$  subunit (Fig. 4). The relative level of the CG $\alpha$  mRNA was quite low in undifferentiated cells, but the relative level was increased 23.9-fold after differentiation. The levels of the CG $\beta$  mRNA, on the other hand, increased only about 2-fold after differentiation for 2 weeks. Minor subpopulations of R278.5 cells differentiated even in the presence of fibroblasts, and the low level of  $\alpha$ -fetoprotein, CG $\alpha$ , and CG $\beta$  mRNA present prior to the removal from fibroblasts could have been from these cells.

All SCID mice injected with R278.5 cells in either intramuscular or intratesticular sites formed tumors, and tumors in both sites demonstrated a similar range of differentiation. The

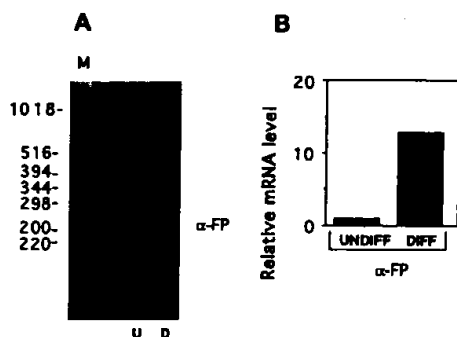


FIG. 3. Expression of  $\alpha$ -fetoprotein mRNA. (A) PCR amplification of  $\alpha$ -fetoprotein ( $\alpha$ FP) cDNA from reverse-transcribed total RNA from undifferentiated (U) and differentiated (D) R278.5 cells. The DNA size markers (M) are indicated in bp. (B) The  $\alpha$ -fetoprotein mRNA levels are expressed relative to the levels of the mRNA for G3PDH in each sample (not shown) as described in the text. Similar results were obtained in a second independent differentiation experiment.

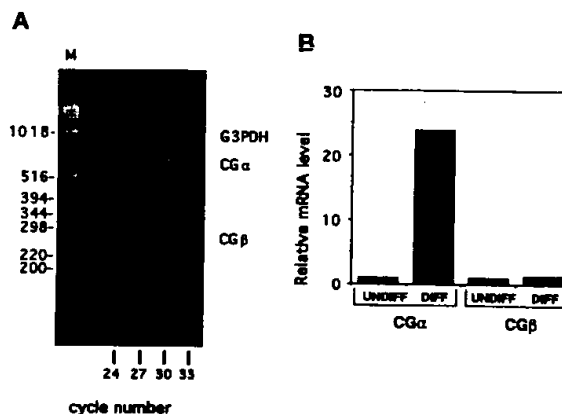


FIG. 4. Expression of CG subunit mRNA. (A) PCR amplification of cDNAs for G3PDH, CG $\alpha$ , and CG $\beta$  subunits from reverse-transcribed total RNA from differentiated R278.5 cells. The DNA size markers (M) are indicated in bp. (B) Relative levels of CG $\alpha$  and CG $\beta$  mRNAs in undifferentiated and differentiated R278.5 cells. Total RNA from cultured cells was analyzed for CG mRNA levels by RT-PCR and expressed relative to the levels of G3PDH mRNA. Similar results were obtained in a second independent differentiation experiment.

oldest tumors examined (15 weeks) had the most advanced differentiation, and all had abundant, unambiguous derivatives of all three embryonic germ layers, including ciliated columnar epithelium and nonciliated columnar epithelium (probable respiratory and gut epithelium; endoderm); bone, cartilage, smooth muscle, striated muscle (mesoderm); ganglia, other neural tissue, and stratified squamous epithelium (ectoderm), and other unidentified cell types (Fig. 5). Neural tissue included stratified cellular structures with remarkable resemblance to developing neural tube (Fig. 5D). Gut-like structures were often encircled by multiple layers of smooth muscle and were sometimes lined by villi with columnar epithelium interspersed with scattered mucus-secreting goblet cells (Fig. 5A and F). Stratified squamous epithelium often contained well-differentiated hair follicles with hair shafts (Fig. 5C).

## DISCUSSION

To our knowledge, there have been no previous reports of the isolation of a primate ES cell line. The characteristics that define R278.5 cells as ES cells include indefinite (>1 year) undifferentiated proliferation *in vitro*, maintenance of a normal karyotype, and potential to differentiate to derivatives of trophoblast and all three embryonic germ layers. The development of complex structures in tumors in SCID mice with remarkable resemblance to normal hair follicles, neural tube, and gut demonstrates the ability of R278.5 cells to participate in complex developmental processes requiring coordinated interactions between multiple cell types. In the mouse embryo, the last cells capable of contributing to derivatives of trophoblast and ICM are early ICM cells of the expanding blastocyst (29). The timing of commitment to ICM or trophoblast has not been established for any primate species, but the potential of R278.5 cells to contribute to derivatives of both suggests that they most closely resemble early totipotent embryonic cells. The very limited ability of mouse ES cells to contribute to trophoblast in chimeras (30) suggests that the R278.5 cells represent an earlier developmental stage than mouse ES cells or that the ability of ICM cells to form trophoblast persists longer in primates. Human EC cells share the ability of R278.5 cells to differentiate to trophoblast *in vitro* (16) and this potential may be a general distinguishing property of primate ES cell lines.

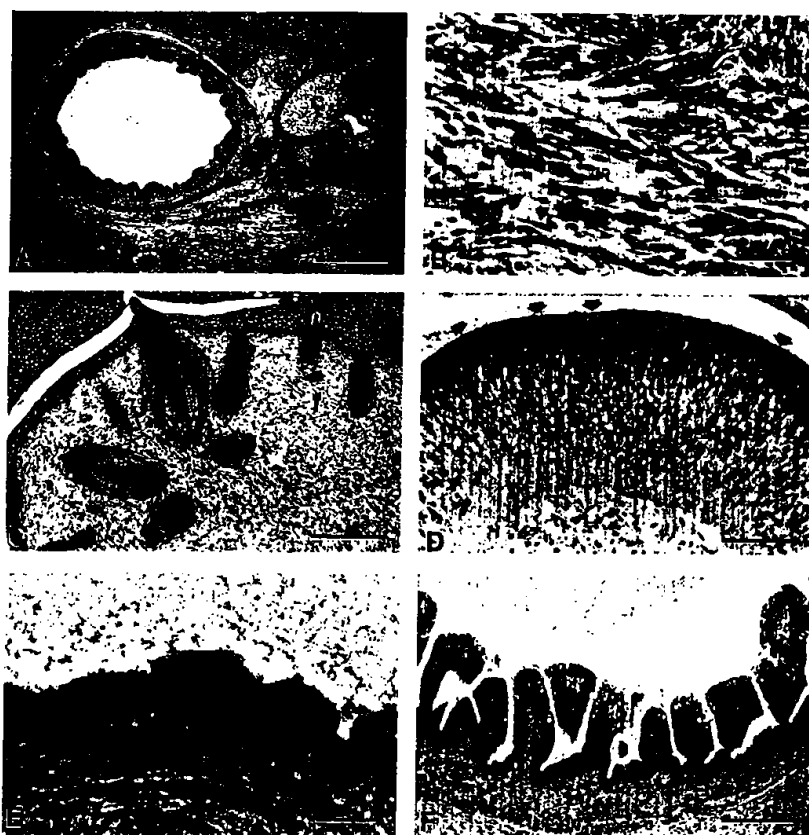


FIG. 5. Tumors formed by R278.5 cells injected into SCID mice and examined at 15 weeks. (A) Low-power field demonstrating disorganized differentiation of multiple cell types. A gut-like structure is circled by smooth muscle (s), and elsewhere foci of cartilage (c) are present. (Bar = 400  $\mu$ m.) (B) Striated muscle. (Bar = 40  $\mu$ m.) (C) Stratified squamous epithelium with several hair follicles. The labeled hair follicle (f) has a visible hair shaft. (Bar = 200  $\mu$ m.) (D) Stratified layers of neural cells in the pattern of a developing neural tube. An upper "ventricular" layer, containing numerous mitotic figures (arrows), overlies a lower "mantle" layer. (Bar = 100  $\mu$ m.) (E) Ciliated columnar epithelium. (Bar = 40  $\mu$ m.) (F) Villi covered with columnar epithelium with interspersed mucus-secreting goblet cells. (Bar = 200  $\mu$ m.)

The only cells known to express the combination of markers alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 other than R278.5 cells are human EC cells (16, 25, 31). This expression pattern contrasts with undifferentiated mouse ES and EC cells, which instead express SSEA-1 and do not express SSEA-3, SSEA-4, TRA-1-60, or TRA-1-81 (23, 24). Differentiation of human EC cells such as NTERA2 cl.D1 (17) results in the loss of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 expression and an increased SSEA-1 expression (16). These antigens have yet to be studied in early human or nonhuman primate embryos, and their functions are unknown, but their shared expression by R278.5 cells and human EC cells suggests a close embryological similarity.

In the absence of fibroblast feeder layers, soluble LIF fails to prevent the differentiation of R278.5 cells or of feeder-dependent human EC cells (19). The factors that fibroblasts produce that prevent the differentiation of R278.5 cells or feeder-dependent human EC cells are unknown. Other factors that fail to support the growth of feeder-dependent human EC cells in the absence of feeder layers include oncostatin M and ciliary neurotrophic factor (19), both of which can substitute for LIF in preventing the differentiation of mouse ES cells (32, 33). A trypsin-sensitive factor from a human yolk sac carcinoma cell line (GCT 44) supports the growth of feeder-dependent human EC cells in the absence of fibroblasts, but the factor has not yet been purified (19).

Although exogenous LIF was added during the initial derivation of R278.5 cells, the cell line is now routinely passaged

without added LIF. We have also recently derived two additional cell lines (R366 and R367) from four additional rhesus blastocysts, using the same techniques as described for R278.5 cells, but without added LIF (data not shown). R366 and R367 cells have normal karyotypes and continue to proliferate *in vitro* for at least 3 months. R366 and R367 cell lines have not yet been tested for tumor formation in SCID mice, but they are indistinguishable from R278.5 cells in undifferentiated morphology, growth characteristics, and *in vitro* differentiation in the absence of feeder layers.

The differentiation of R278.5 cells to trophoblast was demonstrated by the expression of CG $\alpha$  and CG $\beta$  subunit mRNAs and the secretion of bioactive CG into the culture medium by differentiated ES cells. We were surprised to note that while the relative levels of the CG $\alpha$  subunit were increased >20 times in differentiated cells, the relative levels of the CG $\beta$  subunit only changed about 2-fold. The fact that CG secretion increased substantially with differentiation may mean that under our *in vitro* culture conditions, expression of the CG $\alpha$  subunit is limiting for CG secretion. CG $\beta$  subunit mRNA is detectable in human preimplantation embryos as early as the eight-cell stage, which is before trophectoderm differentiation (34), consistent with a low level of CG $\beta$  mRNA expression in undifferentiated R278.5 cells. Although there may be some coordinate mechanisms regulating CG $\alpha$  and CG $\beta$  gene transcription in the placenta (35), it is clear that there is differential regulation of these genes *in vitro* and *in vivo* (36). Since the expression of the CG $\beta$  subunit is also divergent among villous

and extravillous trophoblasts (37), further studies are needed to determine the phenotype of the trophoblasts derived from R278.5 cells.

Primate ES cells will be particularly useful for *in vitro* developmental studies of lineages that differ substantially between humans and mice. However, the most accurate *in vitro* model of the differentiation of human tissues would be provided by human ES cells. In one published report, ICM-derived cells from spare *in vitro* fertilized human embryos were cultured with LIF in the absence of feeder layers, and, although alkaline phosphatase positive cells proliferated, they failed to survive beyond two passages (38). These results suggest that soluble LIF alone will not prevent the differentiation of human ES cells, just as it fails to prevent the differentiation of rhesus ES cells. The growth of rhesus monkey ES cells in culture conditions that support feeder-dependent human EC cells suggests that similar conditions may support human ES cells.

Human ES cells would offer exciting new possibilities for transplantation medicine. Because ES cells have the developmental potential to give rise to all adult cell types, any disease resulting from the failure of specific cell types would be potentially treatable through the transplantation of differentiated cells derived from ES cells. Because ES cells are immortal cell lines, they could be genetically manipulated prior to differentiation either to reduce immunogenicity or to give them new properties to combat specific diseases. Rhesus monkey ES cells and rhesus monkeys will be invaluable for testing the safety and efficacy of the transplantation of specific cell types for the treatment of specific diseases. Because of the range of diseases potentially treatable by this approach, elucidating the basic mechanisms controlling the differentiation of primate ES cells has dramatic clinical significance.

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